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MRI-Based Iron Phenotyping and Patient Selection for Next-Generation Sequencing of Non–Homeostatic Iron Regulator Hemochromatosis Genes

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BACKGROUND AND AIMS: High serum ferritin is frequent among patients with chronic liver disease and commonly associated with hepatic iron overload. Genetic causes of high liver iron include homozygosity for the p.Cys282Tyr variant in homeostatic iron regulator (*HFE*) and rare variants in non-HFE genes. The aims of the present study were to describe the landscape and frequency of mutations in hemochromatosis genes and determine whether patient selection by noninvasive hepatic iron quantification using MRI improves the diagnostic yield of next-generation sequencing (NGS) in patients with hyperferritinemia.

APPROACH AND RESULTS: A cohort of 410 unselected liver clinic patients with high serum ferritin (defined as ≥200 μg/L for women and ≥300 μg/L for men) was investigated by *HFE* genotyping and abdominal MRI R2*. Forty-one (10%) patients were homozygous for the p.Cys282Tyr variant in *HFE*. Of the remaining 369 patients, 256 (69%) had high transferrin saturation (TSAT; ≥45%) and 199 (53%) had confirmed hepatic iron overload (liver R2* ≥70 s^{-1}). NGS of hemochromatosis genes was carried out in 180 patients with hepatic iron overload, and likely pathogenic variants were identified in 68 of 180 (38%) patients, mainly in *HFE* (79%), ceruloplasmin (25%), and transferrin receptor 2 (19%). Low spleen iron (R2* <50 s^{-1}), but not TSAT, was significantly associated with the presence of mutations. In 167 patients (93%), no monogenic cause of hepatic iron overload could be identified.

CONCLUSIONS: In patients without homozygosity for p.Cys282Tyr, coincident pathogenic variants in *HFE* and non-HFE genes could explain hyperferritinemia with hepatic iron overload in a subset of patients. Unlike *HFE* hemochromatosis, this type of polygenic hepatic iron overload presents with variable TSAT. High ferritin in blood is an indicator of the iron storage disease, hemochromatosis. A simple genetic test establishes this diagnosis in the majority of patients affected. MRI of the abdomen can guide further genetic testing. (Hepatology 2021;74:2424-2435).

The concentration of serum ferritin is a surrogate of body iron. Hypoferritinemia is sensitive and specific for iron deficiency.[1] In contrast, hyperferritinemia can be caused by a range of disorders, including iron overload, inflammation, metabolic

Abbreviations: BMI, body mass index; BMP6, bone morphogenetic protein 6; CP, ceruloplasmin; DIOS, dysmetabolic iron overload syndrome; FIB-4, Fibrosis-4 score; GATK, GenomeAnalysisToolkit; HAMP, hepcidin; HFE, homeostatic iron regulator; HIC, hepatic iron concentration; NGS, next-generation sequencing; OR, odds ratio; ROC, receiver operating characteristic; SLC40A1, solute carrier family 40 member 1; TFR2, transferrin receptor 2; TSAT, transferrin saturation.

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syndrome, increased alcohol consumption, and malignancies.[2-5] The diagnostic approach in patients with elevated serum ferritin includes clinical, biochemical, and genetic investigations as well as imaging studies. The sequence of investigations and interpretation of test results is determined by the specific clinical context. Given that the liver is the primary organ for excess iron storage, quantification of hepatic iron is a key step in assessing the cause of hyperferritinemia.[3]

The differential diagnosis of confirmed hepatic iron overload includes repeated transfusions, intravenous or oral iron therapy, homeostatic iron regulator (*HFE*) hemochromatosis, and other inherited disorders of iron metabolism.[6,7] The commonest cause of hepatic iron overload is hemochromatosis, which is genetically heterogeneous and its penetrance modified by environmental factors. In Whites, homozygosity for p.Cys282Tyr in *HFE* accounts for hemochromatosis in 80%-90% of patients with the clinical diagnosis.[2,8-10] Although the frequency of compound heterozygosity for p.Cys282Tyr/p.His63Asp is higher in hemochromatosis patients than in the general population, this genotype alone is not considered sufficient to cause hepatic iron overload.[11] In patients with early disease onset and severe clinical manifestations, recessive mutations in hepcidin (*HAMP*), transferrin receptor 2 (*TFR2*), and hemojuvelin (*HJV*) have been identified.[12,13] In contrast, dominant mutations in solute carrier family 40 member 1 (*SLC40A1*) are associated with iron overload in patients with ferroportin disease or hemochromatosis type 4.[13-16]

Genetic evaluation of patients with hepatic iron overload beyond *HFE* genotyping therefore usually requires sequencing of multiple genes. Recent studies show that next-generation sequencing (NGS) of multiple genes in patients with unexplained iron overload is feasible, but potentially disease-causing variants were identified in only a minority.[17-20] A frequent cause of hepatic iron overload is dysmetabolic iron overload syndrome (DIOS), which is characterized by mild hepatic iron overload associated with metabolic syndrome.[4] NAFLD, with high serum ferritin and normal hepatic iron concentrations, is a condition known as metabolic hyperferritinemia, which is associated with increased risk of liver fibrosis.[21,22]

The presence of fatty liver disease and metabolic syndrome does not rule out an underlying genetic cause of iron overload or high ferritin, but genetic testing beyond *HFE* genotyping is expensive and time-consuming.

Another diagnostic challenge is that cirrhosis is frequently associated with increased hepatic iron storage,[23] which could be a consequence of an impaired liver function or the cause of advanced liver disease. Diagnostic algorithms have been proposed, when to refer patients for genetic testing, but their diagnostic utility is open for study.[24-26]

Hepatic iron overload can result from the reduced production of the iron hormone, hepcidin, in patients with impaired liver function or direct inhibition of hepcidin production by alcohol.[5] Hepcidin is normally produced and secreted by hepatocytes in response to high iron. When circulating hepcidin binds to the iron exporter, ferroportin, expressed in hepatocytes, duodenal enterocytes, and macrophages, ferroportin is internalized and degraded, thereby reducing cellular iron release.[27] Hepcidin deficiency or resistance are the hallmark of hemochromatosis,

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alcoholic siderosis, and iron overload in patients with end-stage liver disease.[28] At early stages, hepcidin deficiency causes high plasma iron and increased transferrin saturation (TSAT) with hepatic iron overload and low-to-normal spleen iron concentration.[29] Although hepcidin concentrations can be reliably quantified in serum and plasma, the clinical utility of measuring plasma hepcidin is also uncertain.[30,31]

The aim of the present study was to assess hepatic iron concentrations by MRI in a cohort of patients with hyperferritinemia and determine the frequency of *HFE* genotypes. NGS was carried out to study the prevalence of variants in non-HFE genes in patients with hepatic iron overload but no evidence of *HFE* hemochromatosis. Each patient underwent a comprehensive panel of clinical and biochemical tests, including serum hepcidin, as well as noninvasive quantification liver stiffness and liver and spleen iron concentration.

Materials and Methods

PATIENT SELECTION AND DATA

A cohort of 451 unselected liver clinic patients referred to the outpatient liver clinic at the University Hospital of Innsbruck (Innsbruck, Austria) from September 1, 2007 to November 30, 2017 for the evaluation of hyperferritinemia (defined as ≥200 μg/L for women and ≥300 μg/L for men) was investigated by *HFE* genotyping and noninvasive liver and spleen iron quantification with R2*-weighted MRI (Fig. 1). The start date of this cohort corresponds to the MRI date. Blood for biochemical and genetic analysis was taken within 4 weeks before or after MRI. After exclusion of 41 patients with history of blood transfusions in the year before inclusion (date of MRI), clinical, biochemical, and radiological data of 410 patients were retrospectively analyzed. For survival analysis, patient

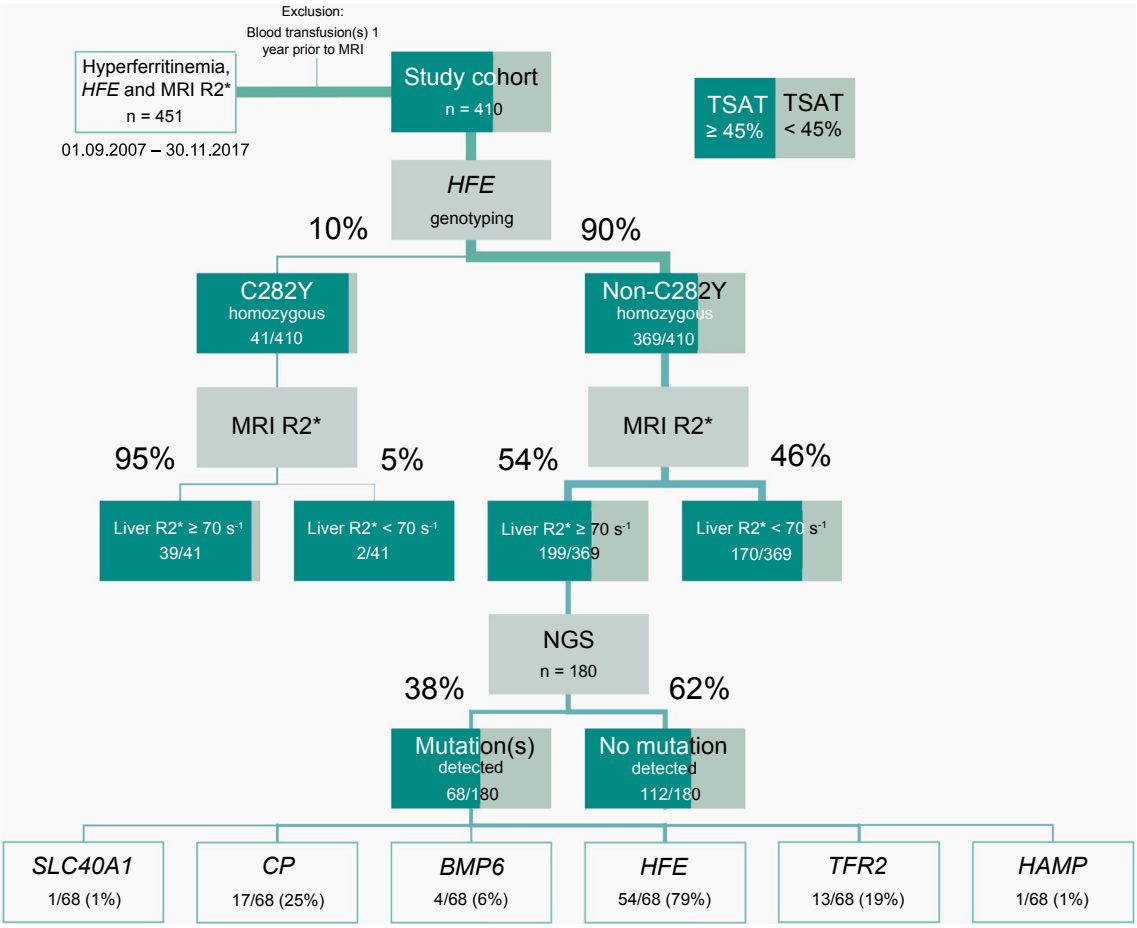

FIG. 1. Study flowchart. Number of patients in different iron phenotype/genotype subgroups. Dual colored boxes show the relative number of patients with TSAT ≥ and < 45%.

status (alive/dead) was assessed by a database query of the National Health Insurance system (*Hauptverband der Sozialversicherungen*) on January 29, 2019. Median time from MRI to death or last patient follow-up was 26 months (4–63; 25th and 75th percentiles). From 199 patients with documented hepatic iron overload (defined as R2* ≥70 sec^{-1}[32,33]), but no *HFE* hemochromatosis, 180 patients had given their written informed consent and were selected for further genetic testing by NGS. We received ethical approval by the ethical committee of the Medical University of Innsbruck to conduct this study (internal registration no.: UN5093).

DEFINITION OF LIVER DISEASE ETIOLOGY

Etiology of liver disease was classified according to the clinical diagnosis extracted from the clinical records and, whenever possible, confirmed with retrospective assessment of histology, laboratory parameters, and imaging data. Patients with alpha-1 antitrypsin deficiency, drug-induced liver injury, or Wilson's disease were classified as "others."

HEPCIDIN 25 ELISA

Hepcidin was measured in 168 patients from whom sera was available using the DRG Hepcidin 25 ELISA kit, according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany).

STATISTICAL ANALYSIS

IBM SPSS Statistics (version 22.0.0.1; IBM Corp., Armonk, NY) and MedCalc software (version 17.7.2; MedCalc Software, Ostend, Belgium) were used for statistical analysis, and a significance value of 0.05 was considered in all statistical tests. The Kolmogorow-Smirnow test was used to test normality of distribution. Not normally distributed variables were reanalyzed after logarithmic transformation. Continuous variables were reported using median and 25th and 75th percentiles and analyzed using the Student *t* test or the Mann Whitney U test, as appropriate. Categorical variables are expressed in frequencies (with percentages in parenthesis) and were tested for significance using the χ^2 test or the Fisher's exact test. Receiver operating characteristic (ROC) calculations were performed, and the Youden index was applied to calculate the optimal cut-off point. Kaplan-Meier analysis was then carried out to determine survival, and groups were compared using the log-rank test. Uni- and multivariate binary logistic regression show the corresponding odds ratios (OR) and 95% CI.

IMAGING PROTOCOL AND ANALYSIS

All patients were examined with a 1.5 Tesla MRI scanner (Magnetom Avanto; Siemens Healthcare, Erlangen, Germany). Patients were scanned in the supine position using a standard anterior six-element body matrix coil and six to nine elements of the integrated spine matrix coil. R2* values were obtained using a fat-saturated multigradient echo sequence with 12 echoes (repetition time = 200 ms; echo time [TE]-initial, 0.99 ms; Delta-TE, 1.41 ms; slice thickness, 10 mm).[34,35] Hepatic iron overload was defined as R2* ≥70 s^{-1},[33,35] and hepatic iron concentrations (HICs) were calculated through the equation: HIC (mg/g) = 0.029 × liver R2* (s^{-1}) + 0.137.[34]

NGS

Library Preparation

Libraries for sequencing were initially prepared using the Nextera DNA Library Prep for Enrichment (Illumina); for enrichment, the xGen Exome Research Panel v1.0 was used (IDT), both according to the manufacturer's recommendations.

Sequencing

Sequencing data were generated on an Illumina HiSeq 4000 instrument (Illumina, San Diego, CA), using a read-length of 2 × 75 bp for the first 38 patients, and, in the remaining 142 patients, NovaSeq S4 was used, using a read length of 2 × 100 bp. On average, ~73× coverage of the exome target was reached per sample, after trimming and filtering.

Variant Calling

Variants were identified using an implementation of the GenomeAnalysisToolkit (GATK) best practice pipeline (version 1.5; https://github.com/marchoeppn

er/exome-seq). Briefly, reads were trimmed (FastP; PMID: 30423086), aligned against the reference genome (hg19; GATK bundle; https://software.broadinstitute.org/gatk/download/bundle), and duplicates marked (GATK 4.1.0.0; PMID: 20644199). Next, read alignments were subjected to several stages of processes to recalibrate base quality scores, generate raw haplotype calls, and perform joint calling of variants across samples and target regions. Finally, variant scores were recalibrated against a set of known truth references (GATK bundle) and filtered to remove variants that did not meet all quality criteria. The resulting call set was annotated for predicted deleterious effects on candidate coding genes using Annovar (September 2018; PMID: 20601685).

Variant Selection

Exonic variants and splice-site variants predicted to have an effect on splicing and/or on the structure and function of a protein were considered pathogenic. As described in http://cadd.gs.washington.edu/score, we selected mutations with a combined annotation-dependent depletion (CADD) score >15, which predicts a functional change on the nucleotide or protein level.[36,37] A gene panel was selected, and variants accounted for further selection (genes in alphabetic order: bone morphogenetic protein 6 [*BMP6*], ceruloplasmin [*CP*], *HAMP*, *HJV*, *HFE*, *SLC40A1*, and *TFR2*).

Results

Patients' baseline characteristics are shown in Supporting Table S1. All patients were genotyped for the p.Cys282Tyr and p.His63Asp polymorphisms in *HFE*. Prevalence of homozygosity for p.Cys282Tyr in this cohort with hyperferritinemia was 10% (41 of 410). Comparison of sex distribution showed that the proportion of females was significantly higher in the subgroup of *HFE* hemochromatosis patients with 37% females as compared to 14% in the group of non-p.Cys282Tyr homozygous patients (Supporting Table S1).

Among *HFE* hemochromatosis patients, 95% (39 of 41) had elevated TSAT (≥45%) and 95% (39 of 41) had hepatic iron overload (R2* ≥70 s^{-1}). In *HFE* hemochromatosis patients, median serum iron and TSAT were significantly higher than in all other patients. Median hepcidin, hepcidin/log(ferritin) ratio, and transferrin concentrations were significantly lower in the *HFE* hemochromatosis patient group. Platelets were significantly lower and Fibrosis-4 score (FIB-4) scores significantly higher in patients without *HFE* hemochromatosis, but median ferritin was not different between groups. One- and 5-year overall survival rates were comparable in both groups (Supporting Table S1).

Median hepatic iron concentration was significantly higher in patients homozygous for p.Cys282Tyr, whereas spleen R2* was significantly lower (Fig. 2B,C). Correlation between liver R2* and TSAT as well as correlation between ferritin and hepcidin in p.Cys282Tyr homozygous patients were distinct from all other *HFE* genotypes (Fig. 2C,D). No such differences were found in correlation analysis between liver R2*/spleen R2* and hepcidin (Fig. 2E,F).

Next, we analyzed patients without *HFE* hemochromatosis (non-p.Cys282Tyr homozygous) and stratified them by the presence of hepatic iron overload (Table 1). Fifty-four percent (199 of 369) of patients without p.Cys282Tyr homozygosity had an increased hepatic iron concentration on R2* MRI. No demographic differences were noted, but patients with hepatic iron overload had a significantly higher prevalence of fatty liver disease as underlying etiology and history of blood transfusions (Supporting Table S2). In the group of patients without hepatic iron overload, median concentration of ferritin and triglycerides were significantly higher and TSAT lower (Supporting Table S2). Genetically, compound hetero- and homozygosity for the p.His63Asp polymorphism were significantly more common in the group of patients with hepatic iron overload (Supporting Fig. S1). Noninvasive fibrosis markers (FIB-4 and aspartate transaminase to platelet radio index score [APRI]) were significantly lower in patients with hepatic iron overload than in patients with high ferritin but normal hepatic iron concentration. One- and 5-year overall survival rates did not differ (Table 1).

In order to determine the frequency and landscape of mutations in *HFE* and non-HFE hemochromatosis genes in patients with hepatic iron overload but no *HFE* hemochromatosis, NGS was carried out in 180 patients (Supporting Tables 2 and S3). Thirty-eight percent (68 of 180) of patients had potentially disease-associated variants in hemochromatosis genes

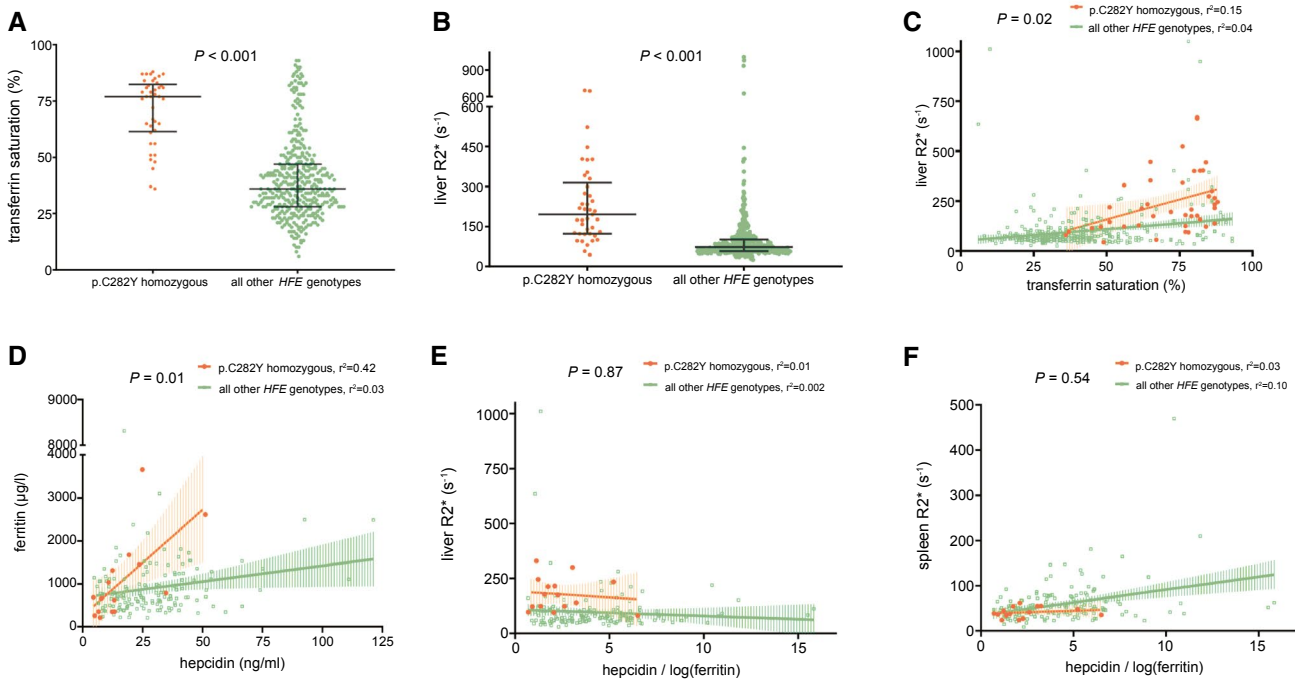

FIG. 2. Serum iron parameters, tissue iron concentrations and correlation analysis in individual patients stratified by HFE genotype. (A) Transferrin saturation is significantly higher in p.C282Y homozygous in comparison with all other HFE genotypes, defining the first step on the diagnostic algorithm for hemochromatosis. (B) Liver R2* as a surrogate of HIC is also significantly higher in p.C282Y homozygous in comparison with other HFE genotypes. (C, D) Patients homozygous for the p.C282Y mutation show a significantly stronger correlation between liver R2* and transferrin saturation (C) and between ferritin and hepcidin concentrations (D). (E, F) No significant differences where observed in thecorrelation between liver R2* (E) and spleen R2* (F) with hepcidin/log(ferritin).

TABLE 1. Clinical, Biochemical, and Radiological Data of Hyperferritinemia Patients Without p.Cys282Tyr Homozygosity Stratified by Hepatic Iron Overload

	Non-p.Cys282Tyr HH (n = 369)		
	Hepatic Iron Overload (n = 199)	No Hepatic Iron Overload (n = 170)	*P* Value
Age, years	55 (45-66)	52 (43-63)	0.02
Sex, female/male (%)	29/170 (15/85)	24/146 (14/86)	0.90
BMI, kg/m^2	26.3 (23.1-29.6)	27.8 (24.6-30.2)	0.05
Transfusion history, n (%)	21 (11)	6 (4)	0.01
Cirrhosis, n (%)	18 (9)	11 (6)	0.36
FIB-4	1.78 (1.17-2.50)	2.00 (1.25-3.17)	0.02
Serum iron, µmol/L	22.4 (17.8-28.6)	21.5 (17.1-27.9)	0.39
Serum ferritin, µg/L	874 (599-1,242)	559 (426-761)	<0.001
Transferrin, mg/dL	231 (202-256)	249 (225-279)	<0.001
TSAT, %	40 (30-50)	34 (28-44)	0.008
Hepcidin/log(ferritin)	4.2 (2.6-5.7)*	3.6 (2.3-5.7)**	0.26
Liver R2*, s^{-1}	97.4 (77.9-138.7)	56.5 (49.5-63.0)	<0.001
HIC, mg/g	3.0 (2.4-4.2)	1.8 (1.6-2.0)	<0.001
Spleen R2*, s^{-1}	57.0 (42.3-78.2)	42.9 (32.1-52.8)	<0.001
1-year overall survival rate, % (n)	97.9 (137/140)	99.1 (111/112)	0.63
5-year overall survival rate, % (n)	84.0 (68/81)	82.0 (41/50)	0.81

Data are given as n (%) or median (interquartile range); *n = 83, **n = 70.
Abbreviation: HH, hereditary hemochromatosis.

TABLE 2. Clinical, Biochemical, and Radiological Data of Patients Analyzed by NGS

	Mutation(s) Detected (n = 68)	No Mutation Detected (n = 112)	*P* Value
Age, years	56 (42-67)	55 (47-65)	0.61
Sex, female/male (%)	11/57 (16/84)	17/95 (15/85)	0.86
BMI, kg/m^2	26.6 (22.8-29.6)	26.2 (23.3-29.4)	0.68
Transfusion history, n (%)	5 (7)	15 (13)	0.21
Cirrhosis, n (%)	3 (4)	14 (13)	0.07
FIB-4	1.6 (1.0-2.3)	1.8 (1.2-2.5)	0.16
Serum iron, µmol/L	24.6 (18.9-29.2)	21.2 (16.4-28.1)	0.06
Serum ferritin, µg/L	797 (544-1,137)	936 (643-1,396)	0.03
Transferrin, mg/dL	225 (198-248)	231 (199-259)	0.41
TSAT, %	43 (35-50)	36 (28-52)	0.04
C-reactive protein, mg/dL	0.12 (0.06-0.29)	0.19 (0.10-0.40)	0.04
Hepcidin/log(ferritin)	3.5 (1.9-5.1)*	4.9 (3.7-6.1)**	0.007
Liver R2*, s^{-1}	106.6 (79.7-131.6)	95.3 (77.0-149.6)	0.59
HIC, mg/g	3.2 (2.4-4.0)	2.9 (2.4-4.5)	0.59
Spleen R2*, s^{-1}	51.5 (38.4-70.3)	63.4 (48.4-90.0)	0.002
1-year overall survival rate, % (n)	97.8 (45/46)	97.4 (76/78)	1.00
5-year overall survival rate, % (n)	86.4 (19/22)	79.5 (35/44)	0.74

Data are given as n (%) or median (interquartile range); *n = 33, **n = 48.

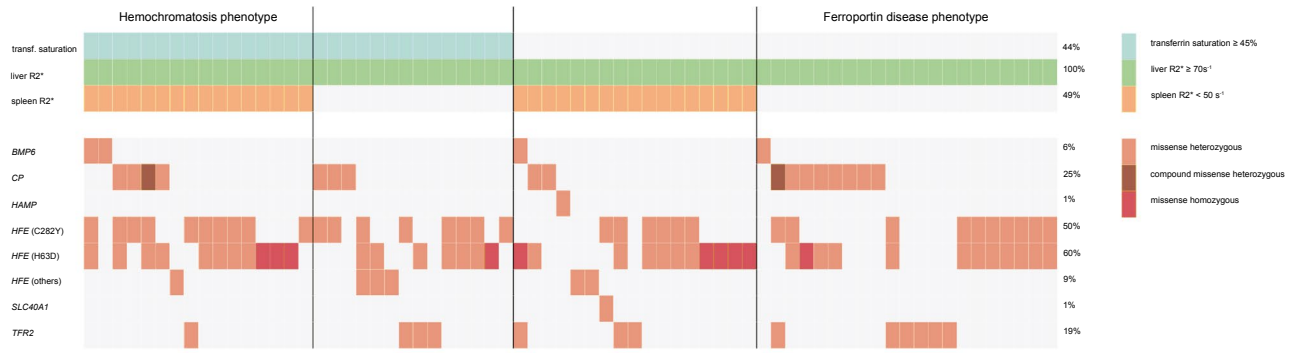

FIG. 3. NGS results. *Top*: transferrin saturation, liver and spleen R2*, *central heatmap*: distributionof mutations in the selected genes, and *percentages on the right side*: overall mutation frequency for each gene.

(*BMP6*, *CP*, *HAMP*, *HFE*, *SLC40A1*, and *TFR2*; Fig. 3). In 8 of all 180 sequenced patients (4%), a monogenetic diagnosis could be made. Specifically, biallelic variants were found in *CP* in 2 patients, one of whom had additional variants in *HFE* and *TFR2*. One additional patient had a rare *HFE* variant in combination with p.Cys282Tyr. In 5 patients (3%), likely pathogenic variants were found in genes causing autosomal-dominant hemochromatosis (*BMP6* and *SLC40A1*). In the remaining 167 patients (93%), no monogenic cause of hepatic iron overload could be identified. In search for polygenic causes of hepatic iron overload, likely pathogenic heterozygous variants in two hemochromatosis genes were recorded. Nine of these 167 patients (5%) were heterozygous for p.Cys282Tyr in *HFE* and had an additional likely pathogenic variant in a non-HFE gene (Fig. 3). In 46 of the remaining 158 patients (29%), a variant was either present in a single heterozygous state or in combination with p.His63Asp in *HFE* (Fig. 3; Supporting Table S4).

In the group of patients with mutations in *HFE* and non-HFE genes, serum ferritin was significantly lower and TSAT higher than in patients without likely pathogenic variants. Hepcidin/log(ferritin) ratio

and spleen R2* were lower in patients with mutations detected (Fig. 4A; Table 2). Binary logistic regression analysis identified low spleen iron and low serum ferritin, but not TSAT, as independent predictors of likely disease-associated variants (Table 3). Further ROC curve analysis on spleen R2* revealed the highest Youdex index for a cutoff of 50 s^{-1} (Fig. 4B). No significant differences in 1- and 5-year survival were present (Supporting Fig. S2).

Finally, phenotypic and genetic characteristics of patients with suspected hemochromatosis were compared. For this aim, likely secondary causes of hepatic iron overload were excluded and patients were grouped by TSAT. Prevalence of hepatic iron overload was 77% in patients with elevated TSAT and 65% in those with TSAT <45% (Fig. 5), but this difference did not reach statistical significance (P = 0.14, Fisher's exact test). The mutation detection rate was 46% in patients with high TSAT and 40% in those who presented with a TSAT <45% (P = 0.7, Fisher's exact test), and the spectrum of likely pathogenic variants in hemochromatosis genes was similar (Fig. 5).

Discussion

The results presented here show the diagnostic yield of *HFE* genotyping, noninvasive assessment of hepatic and splenic iron by MRI, serum hepcidin concentration, and NGS in liver clinic patients with hyperferritinemia.

The hemochromatosis phenotype with hepatic iron overload and high TSAT was present in 22% (89 of 410) of all patients, of whom 43% (38 of 89) were homozygous for p.Cys282Tyr. Current guidelines recommend performing *HFE* genotyping as the first diagnostic step in patients with high serum ferritin and elevated TSAT.[24,26] When patient selection for *HFE* genotyping was based on ferritin and TSAT alone, the diagnostic yield was 15% (39 of 258) in our cohort. Further

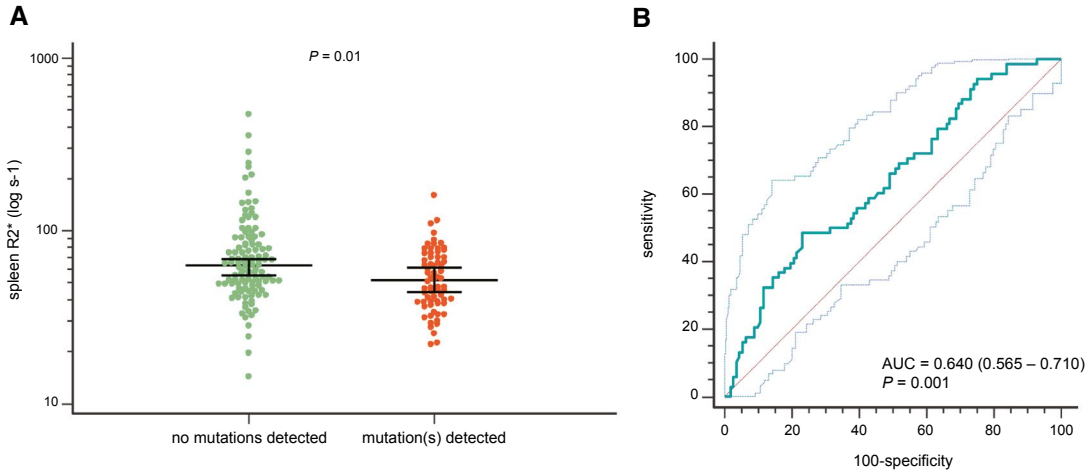

FIG. 4. Spleen R2* as a selecting criterion for NGS. (A) In the subgroup of patients who were further studied through NGS (n = 180), spleen R2* values were significantly lower in patients where probably disease-associated mutations were found. (B) ROC curve analysis showed an AUC of 0.64 (95% confidence interval between parentheses), with the highest Youdex index for a cut-off of 50 s^{-1} (sensitivity 49%, specificity 77%).

TABLE 3. Binary Logistic Regression to Estimate the Risk for Detection of Non-*HFE* Mutations (Defined as Non-p.Cys282Tyr Homozygous) by NGS

	OR Univariate (95% CI)	*P* Value	OR Multivariate (95% CI)	*P* Value
Serum ferritin, µg/L	0.999 (0.999 – 1.000)	0.04	0.999 (0.999-1.000)	0.46
TSAT, %	1.008 (0.993 – 1.024)	0.30		
C-reactive protein, mg/dL	1.065 (0.884 – 1.283)	0.51		
Hepcidin/log(ferritin)	0.839 (0.694 – 1.014)	0.07		
Spleen R2*, s^{-1}	0.983 (0.972 – 0.995)	0.004	0.985 (0.973-0.997)	0.02

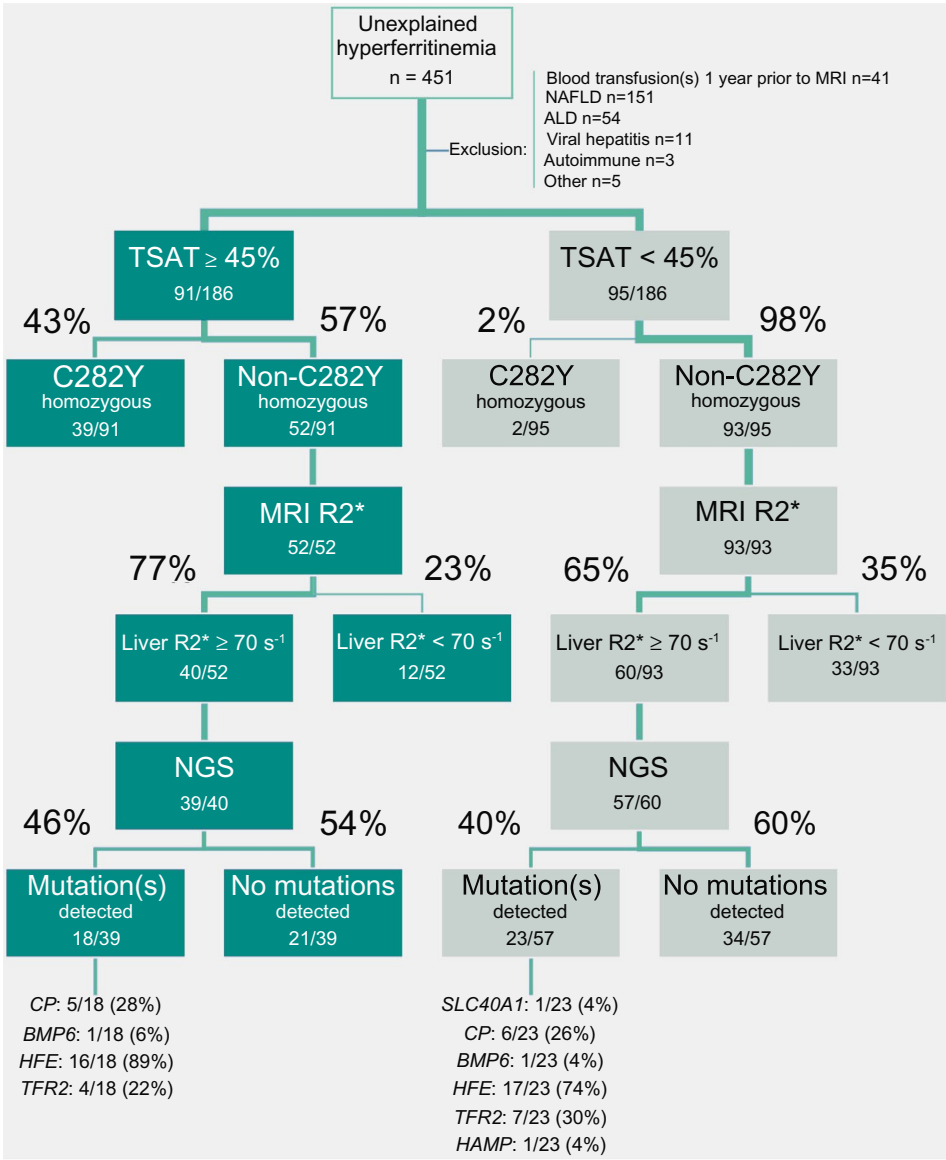

FIG. 5. Prevalence of hepatic iron overload and hemochromatosis gene variants after exclusion of likely secondary causes of hyperferritinemia and according to TSAT. Other: alpha-1antitrypsin deficiency, drug induced liver injury or Wilson disease.

testing for non-*HFE* hemochromatosis is only recommended in patients with hepatic iron overload on MRI or liver biopsy. Hepcidin/log(ferritin) ratio and transferrin concentrations were significantly lower in the *HFE* hemochromatosis patient group. These findings confirm that an elevated TSAT and inappropriately low concentration of hepcidin are the distinctive biochemical phenotypes of *HFE*-associated hemochromatosis (Fig. 2).

Beyond guideline recommendations, MRI was performed in the entire cohort regardless of TSAT. This analysis showed that 62% (71 of 113) of patients with normal TSAT had high liver iron concentrations. TSAT therefore is a good selection marker for *HFE* genotyping, but a poor predictor of hepatic iron overload, in non-p.Cys282Tyr homozygous patients.

In our cohort, patients without hepatic iron overload had a higher body mass index (BMI) and triglycerides, suggesting that NAFLD is a common cause of hyperferritinemia.[38,39] In the case of metabolic hyperferritinemia, ferritin likely reflects subclinical inflammation

and can indicate advanced liver fibrosis.[40] If hyperferritinemia is associated with hepatic iron overload in patients with metabolic syndrome, the condition is known as DIOS.[4] Accordingly, we found higher noninvasive fibrosis scores (FIB-4 and APRI) in metabolic hyperferritinemia than in DIOS. The findings from recent prospective clinical trials, that phlebotomy is ineffective in metabolic hyperferritinemia and DIOS, further suggest that fibrosis is primarily driven by inflammation rather than by iron overload.[41,42]

Secondary causes of high serum ferritin and mildly increased hepatic iron concentrations also include alcohol related liver disease, viral hepatitis, and advanced liver disease, which are all associated with reduced hepcidin levels.[43,44] Given that these conditions can coincide with genetic defects associated with hemochromatosis, patients with hyperferritinemia were included regardless of their risk-factor profile and severity of hepatic iron overload for the primary analysis presented here. After exclusion of patients with potential secondary causes of hyperferritinemia, the mutation detection rate was 43%, which is only slightly higher than in the entire cohort (38%; Fig. 5). This illustrates the genetic contribution to the phenotypic presentation even in patients with acquired cofactors for the development of hyperferritinemia and hepatic iron overload.

High liver iron was present in 54% (199 of 369) of patients without p.Cys282Tyr homozygosity, of whom 65% (129 of 199) had a hemochromatosis phenotype with high TSAT. When patients with hepatic iron overload were evaluated by NGS, regardless of TSAT, potentially disease-causing variants were detected in 38% (68 of 180), but monogenic or digenic causes of hepatic iron overload could be found in only a minority. Therefore, liver iron concentrations could be determined by variants in other genes, common polymorphisms, nutrition, and environmental modifiers.[45]

In *CP*, where most variants were identified after *HFE*, likely pathogenic variants were present in 25% (17 of 68) of patients, 2 of whom were compound heterozygotes. Both patients had normal ceruloplasmin concentrations and no neurological impairment, suggesting that *CP* variants can not only present as aceruloplasminemia, but also with isolated hepatic iron overload. Further *in vitro* studies are needed to determine the functional consequence of these rare variants. Our findings are in line with another study reporting a high prevalence of *CP* variants in patients with hepatic iron overload.[46]

In contrast to previous studies reporting a prevalence of 80%-90% homozygosity for the p.Cys282Tyr variant in *HFE* in Whites with phenotypic hemochromatosis,[8-10] the present study shows that only 10% of patients referred for the evaluation of high serum ferritin were homozygous for this variant. This difference could be attributed to the fact that first-level *HFE* genotyping was performed regardless of increased TSAT and to the distinct phenotype of patients with *BMP6* and *SLC40A1* variants.

Identification of previously reported *BMP6* and *SLC40A1* variants in this study further supports the phenotypic description of non-*HFE* hemochromatosis caused by mutations in these genes.[47,48] The variant, p.L69P, in *BMP6* was found in an 82-year-old patient who had documented normal serum iron parameters at the age of 73 years (Supporting Table S4, patient number 2). At the age of 82, the patient presented with a serum ferritin of 543 μg/L and a hepatic iron concentration of 46 μmol/g (hepatic iron index, 0.56), which indicates a mild phenotype in accord with the previous report on this variant.[47] Patient number 48 (Supporting Table S4) was heterozygous for another likely pathogenic *BMP6* variant and showed a mild phenotype with normal TSAT and a hepatic iron concentration of 37 μmol/g (hepatic iron index, 0.60) and reported a regular alcohol consumption of 50 g/d. These findings suggest that heterozygosity for *BMP6* variants confers only moderate iron overload. The potential disease-modifying effect of *BMP6* variants is indicated by 2 additional patients who were also carriers of biallelic *HFE* polymorphisms (1 compound heterozygous p.Cys282Tyr/p.His63Asp and 1 homozygous for p.His63Asp). One of these patients (Supporting Table S4, patient number 31) even had an additional mutation in *TFR2*. Patient number 37 presented at the age of 35 years and was heterozygous for an *SLC40A1* variant and also compound heterozygous for p.Cys282Tyr/p.His63Asp. His mild iron overload with an HIC of 37 μmol/g (hepatic iron index, 1.1) and normal spleen iron concentration show the age dependence of p.W158C ferroportin disease penetrance.[48]

Potential limitations of the current study include that the functional consequence of sequence variants was predicted by CADD score alone without segregation studies or functional analysis.[36,37] Classification

of variants according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology criteria will therefore require further *in vitro* analysis and independent clinical validation.[49]

Considering the cost and low diagnostic yield of NGS in this real-life clinical cohort, further selection criteria for advanced genetic testing are required. The recent definition of hemochromatosis as a syndrome of low hepcidin explains reduced spleen iron because of uncontrolled iron release.[50] The present study shows that patient selection based on low spleen iron could improve the pretest likelihood for identifying pathogenic variants on NGS. Given that lower spleen iron indicates reduced hepcidin effect, hepcidin was also evaluated for its value to predict pathogenic sequence variants, but did not further improve pretest likelihood.

In conclusion, when approaching liver clinic patients with hyperferritinemia, our data confirm that *HFE* genotyping should be guided by TSAT. High liver iron and low spleen iron are predictors of likely pathogenic variants in hemochromatosis genes. Such recessive variants could explain hyperferritinemia with hepatic iron overload in a subset of patients, who can present with variable TSAT.

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REFERENCES

1) Camaschella C. Iron deficiency. Blood 2019;133:30-39.
2) Allen KJ, Gurrin LC, Constantine CC, Osborne NJ, Delatycki MB, Nicoll AJ, et al. Iron-overload-related disease in HFE hereditary hemochromatosis. N Engl J Med 2008;358:221-230.
3) Koperdanova M, Cullis JO. Interpreting raised serum ferritin levels. BMJ 2015;351:h3692.
4) Moirand R, Mortaji AM, Loreal O, Paillard F, Brissot P, Deugnier Y. A new syndrome of liver iron overload with normal transferrin saturation. Lancet 1997;349:95-97.
5) Whitfield JB, Zhu G, Heath AC, Powell LW, Martin NG. Effects of alcohol consumption on indices of iron stores and of iron stores on alcohol intake markers. Alcohol Clin Exp Res 2001;25:1037-1045.
6) El-Beshlawy A, Inusa B, Beneitez Pastor D, Xicoy B, Soledada Duran Nieto M, Bruederle A, et al. International sentinel site surveillance of patients with transfusional hemosiderosis treated with deferasirox in actual practice setting. Hematology 2019;24:238-246.
7) Tsironi M, Polonifi K, Deftereos S, Farmakis D, Andriopoulos P, Moyssakis I, et al. Transfusional hemosiderosis and combined chelation therapy in sickle thalassemia. Eur J Haematol 2005;75:355-358.
8) Bacon BR, Britton RS. Clinical penetrance of hereditary hemochromatosis. N Engl J Med 2008;358:291-292.
9) Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G–> A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet 2002;359:211-218.
10) Grosse SD, Gurrin LC, Bertalli NA, Allen KJ. Clinical penetrance in hereditary hemochromatosis: estimates of the cumulative incidence of severe liver disease among HFE C282Y homozygotes. Genet Med 2018;20:383-389.
11) Gurrin LC, Bertalli NA, Dalton GW, Osborne NJ, Constantine CC, McLaren CE, et al. HFE C282Y/H63D compound heterozygotes are at low risk of hemochromatosis-related morbidity. Hepatology 2009;50:94-101.
12) Barton JC, Lafreniere SA, Leiendecker-Foster C, Li H, Acton RT, Press RD, et al. HFE, SLC40A1, HAMP, HJV, TFR2, and FTL mutations detected by denaturing high-performance liquid chromatography after iron phenotyping and HFE C282Y and H63D genotyping in 785 HEIRS Study participants. Am J Hematol 2009;84:710-714.
13) Wang Y, Du Y, Liu G, Guo S, Hou BO, Jiang X, et al. Identification of novel mutations in HFE, HFE2, TfR2, and SLC40A1 genes in Chinese patients affected by hereditary hemochromatosis. Int J Hematol 2017;105:521-525.
14) Pietrangelo A. Ferroportin disease: pathogenesis, diagnosis and treatment. Haematologica 2017;102:1972-1984.
15) De Domenico I, Ward DM, Musci G, Kaplan J. Iron overload due to mutations in ferroportin. Haematologica 2006;91:92-95.
16) Viveiros A, Schaefer B, Tilg H, Zoller H. Iron matryoshka-haemochromatosis nested in ferroportin disease? Liver Int 2019;39:1014-1015.
17) Badar S, Busti F, Ferrarini A, Xumerle L, Bozzini P, Capelli P, et al. Identification of novel mutations in hemochromatosis genes by targeted next generation sequencing in Italian patients with unexplained iron overload. Am J Hematol 2016;91:420-425.
18) Faria R, Silva B, Silva C, Loureiro P, Queiroz A, Fraga S, et al. Next-generation sequencing of hereditary hemochromatosis-related genes: novel likely pathogenic variants found in the Portuguese population. Blood Cells Mol Dis 2016;61:10-15.
19) McDonald CJ, Ostini L, Wallace DF, Lyons A, Crawford DH, Subramaniam VN. Next-generation sequencing: application of a novel platform to analyze atypical iron disorders. J Hepatol 2015;63:1288-1293.
20) Uguen K, Scotet V, Ka C, Gourlaouen I, L'Hostis C, Merour MC, et al. Diagnostic value of targeted next-generation sequencing in suspected hemochromatosis patients with a single copy of the HFE p.Cys282Tyr causative allele. Am J Hematol 2017;92:E664-E666.
21) Datz C, Muller E, Aigner E. Iron overload and non-alcoholic fatty liver disease. Minerva Endocrinol 2017;42:173-183.
22) Murali AR, Gupta A, Brown K. Systematic review and meta-analysis to determine the impact of iron depletion in dysmetabolic

iron overload syndrome and non-alcoholic fatty liver disease. Hepatol Res 2018;48:E30-E41.
23) Ludwig J, Hashimoto E, Porayko MK, Moyer TP, Baldus WP. Hemosiderosis in cirrhosis: a study of 447 native livers. Gastroenterology 1997;112:882-888.
24) Bacon BR, Adams PC, Kowdley KV, Powell LW, Tavill AS; American Association for the Study of Liver Diseases. Diagnosis and management of hemochromatosis: 2011 Practice Guideline by the American Association for the Study of Liver Diseases. Hepatology 2011;54:328-343.
25) Brissot P, Pietrangelo A, Adams PC, de Graaff B, McLaren CE, Loréal O. Haemochromatosis. Nat Rev Dis Primers 2018;4:18016.
26) European Association for the Study of the Liver. EASL clinical practice guidelines for HFE hemochromatosis. J Hepatol 2010;53:3-22.
27) Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. Am J Physiol Gastrointest Liver Physiol 2006;290:G199-G203.
28) Pietrangelo A. Genetics, genetic testing, and management of hemochromatosis: 15 years since hepcidin. Gastroenterology 2015;149:1240-1251.e4.
29) Pietrangelo A, Trautwein C. Mechanisms of disease: the role of hepcidin in iron homeostasis—implications for hemochromatosis and other disorders. Nat Clin Pract Gastroenterol Hepatol 2004;1:39-45.
30) Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. Blood 2016;127:2809-2813.
31) Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-1669.
32) Pepe A, Lombardi M, Positano V, Cracolici E, Capra M, Malizia R, et al. Evaluation of the efficacy of oral deferiprone in beta-thalassemia major by multislice multiecho T2*. Eur J Haematol 2006;76:183-192.
33) Schwenzer NF, Machann J, Haap MM, Martirosian P, Schraml C, Liebig G, et al. T2* relaxometry in liver, pancreas, and spleen in a healthy cohort of one hundred twenty-nine subjects-correlation with age, gender, and serum ferritin. Invest Radiol 2008;43:854-860.
34) Henninger B, Zoller H, Rauch S, Finkenstedt A, Schocke M, Jaschke W, et al. R2* relaxometry for the quantification of hepatic iron overload: biopsy-based calibration and comparison with the literature. Rofo 2015;187:472-479.
35) Henninger B, Alustiza J, Garbowski M, Gandon Y. Practical guide to quantification of hepatic iron with MRI. Eur Radiol 2020;30:383-393.
36) Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 2014;46:310-315.
37) Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. Nucleic Acids Res 2019;47:D886-D894.
38) Eslam M, Sanyal AJ, George J; International Consensus Panel. MAFLD: a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. Gastroenterology 2020;158:1999-2014.e1.
39) Fargion S, Mattioli M, Ludovica Fracanzani A, Sampietro M, Tavazzi D, Fociani P, et al. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. Am J Gastroenterol 2001;96:2448-2455.
40) Dongiovanni P, Fracanzani AL, Fargion S, Valenti L. Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. J Hepatol 2011;55:920-932.
41) Adams LA, Crawford DH, Stuart K, House MJ, St. Pierre TG, Webb M, et al. The impact of phlebotomy in nonalcoholic fatty liver disease: a prospective, randomized, controlled trial. Hepatology 2015;61:1555-1564.
42) Lainé F, Ruivard M, Loustaud-Ratti V, Bonnet F, Calès P, Bardou-Jacquet E, et al. Metabolic and hepatic effects of bloodletting in dysmetabolic iron overload syndrome: a randomized controlled study in 274 patients. Hepatology 2017;65:465-474.
43) Czaja AJ. Review article: iron disturbances in chronic liver diseases other than haemochromatosis—pathogenic, prognostic, and therapeutic implications. Aliment Pharmacol Ther 2019;49:681-701.
44) Vela D. Low hepcidin in liver fibrosis and cirrhosis; a tale of progressive disorder and a case for a new biochemical marker. Mol Med 2018;24:5.
45) Wilman HR, Parisinos CA, Atabaki-Pasdar N, Kelly M, Thomas EL, Neubauer S, et al. Genetic studies of abdominal MRI data identify genes regulating hepcidin as major determinants of liver iron concentration. J Hepatol 2019;71:594-602.
46) Corradini E, Bernardis I, Dongiovanni P, Buzzetti E, Caleffi A, Artuso L, et al. Rare ceruloplasmin variants are associated with hyperferritinemia and increased hepatic iron in NAFLD patients: results from a NGS study. J Hepatol 2018;68:58-59.
47) Daher R, Kannengiesser C, Houamel D, Lefebvre T, Bardou-Jacquet E, Ducrot N, et al. Heterozygous mutations in BMP6 pro-peptide lead to inappropriate hepcidin synthesis and moderate iron overload in humans. Gastroenterology 2016;150:672-683.e4.
48) Mayr R, Griffiths WJH, Hermann M, McFarlane I, Halsall DJ, Finkenstedt A, et al. Identification of mutations in SLC40A1 that affect ferroportin function and phenotype of human ferroportin iron overload. Gastroenterology 2011;140:2056-2063, 2063.e1.
49) Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-424.
50) Pietrangelo A. Hemochromatosis: an endocrine liver disease. Hepatology 2007;46:1291-1301.

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